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Identification of amino acid residues that are crucial for FXIII-A intersubunit interactions and stability

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Abstract:
Coagulation factor XIII (FXIII) is the main stabilizer of the fibrin clot. It circulates in plasma as a tetramer of two A and two B subunits. Under physiological conditions, FXIII-A exists as a dimer (FXIII-A_2). The interactions between the FXIII-A subunits that stabilize the FXIII-A_2 dimer are not fully understood. Therefore, we designed a systematic approach to identify amino acid residues crucial for the expression and stability of FXIII-A_2. Based on the available FXIII-A_2 crystal structure, we identified 12 amino acid residues forming intersubunit salt bridges and 21 amino acid residues forming hydrogen bonds between the two A-subunits. We chose ten amino acid residues that form five particularly strong interactions, performed site-directed mutagenesis and expressed the mutants in CHO cells. Disruption of these interactions by single mutation of Lys257, Lys113, Asp343, Glu401, or Asp404 abolished the expression of properly folded, soluble and functional FXIII-A in CHO cells. On the contrary, mutation of Glu111, Arg100, or Asn112 had no significant effect on FXIII-A expression. Our results suggest that four intersubunit interactions (Arg11-Asp343, Lys113-Asp367, Lys257-Glu401 and Arg260-Asp404) are essential for the stability of FXIII-A_2. Our findings are supported by reported mutations at Lys257, Arg260, and Asp404 found in patients with congenital FXIII-A deficiency.

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Identification of amino acid residues that are crucial for FXIII-A intersubunit interactions and stability

Short title: FXIII-A intersubunit interactions

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Key Points

- Targeted mutations of amino acid residues involved in FXIII-A subunit interactions affect FXIII-A expression and activity.
- We have identified eight amino acid residues forming four intersubunit interactions that may be essential for FXIII-A_2 dimer stability.

Abstract

Coagulation factor XIII (FXIII) is the main stabilizer of the fibrin clot. It circulates in plasma as a tetramer of two A and two B subunits. Under physiological conditions, FXIII-A exists as a dimer (FXIII-A_2). The interactions between the FXIII-A subunits that stabilize the FXIII-A_2 dimer are not fully understood. Therefore, we designed a systematic approach to identify amino acid residues crucial for the expression and stability of FXIII-A_2. Based on the available FXIII-A_2 crystal structure, we identified 12 amino acid residues forming intersubunit salt bridges and 21 amino acid residues forming hydrogen bonds between the two A-subunits. We chose ten amino acid residues that form five particularly strong interactions, performed site-directed mutagenesis and expressed the mutants in CHO cells. Disruption of these interactions by single mutation of Lys257, Lys113, Asp343, Glu401, or Asp404 abolished the expression of properly folded, soluble and functional FXIII-A in CHO cells. On the contrary, mutation of Glu111, Arg100, or Asn112 had no significant effect on FXIII-A expression. Our results suggest that four intersubunit interactions (Arg11-Asp343, Lys113-Asp367, Lys257-Glu401 and Arg260-Asp404) are essential for the stability of FXIII-A_2. Our findings are supported by reported mutations at Lys257, Arg260, and Asp404 found in patients with congenital FXIII-A deficiency.

Introduction

Coagulation factor XIII (FXIII) keeps blood clots stable by crosslinking fibrin polymers and protecting fibrin clots from fibrinolytic degradation. It is essential to form a stabilized clot during coagulation to stop bleeding. Deficiency of FXIII causes bleeding manifestations, including potentially life-threatening intracranial bleeding.[1] FXIII-A is a protransglutaminase in a transglutaminase (TG) family of nine proteins. Eight members of the family can exist as monomers, including TG1-TG7 and human erythrocyte membrane protein band 4.2, while FXIII-A only exists as a dimer *in vivo.*[2,3] Plasma FXIII circulates in plasma as a heterotetramer (FXIII-A_2B_2) of two catalytic A-subunits (FXIII-A) and two carrier/regulatory B-subunits (FXIII-B).[4] The catalytic A-subunit contains the transglutaminase active site (Cys314) and is the functional subunit of FXIII.[2] The B-subunit is a carrier protein that stabilizes FXIII-A_2 in plasma.[5] Cellular FXIII is a homodimer of two catalytic A-subunits (FXIII-A_2) present in the cytoplasm of certain cells.[5] No non-activated monomeric FXIII-A is found *in vivo.*[2,5]

FXIII-A consists of 731 amino acids, without the initiator methionine, and has five structural domains: activation peptide (amino acids 1-37), β-sandwich (amino acids 38-184), core domain (amino acids 185-515), β-barrel 1 (amino acids 516-628), and β-barrel 2 (amino acids 629-731). Two identical FXIII-A subunits assemble the dimer (FXIII-A_2).[5] Because there is no covalent bond between two FXIII-A subunits, dimer formation is ensured by non-covalent interactions including salt bridges, hydrogen bonds (H-bonds), hydrophobic interactions, and van der Waals forces. The salt bridge is the strongest interaction among the non-covalent interactions.[6,7] A salt bridge is the interaction between two ionized molecules (also called ion-pairs), defined as a bond formed by an acid and a base with close donor-acceptor pKa matching.[8] In proteins, salt bridges occur between amino acids with opposite fullelectron charges and are in fact a combination of two non-covalent interactions, ionic and hydrogen bonding. A salt bridge is generally considered to exist when the distance between centers of charge are less than 4 Å.[9] The H-bond is another important interaction that maintains protein structure and protein folding. The binding strength of H-bonds is weaker than salt bridges, but stronger than van der Waals forces.[6] The distance between H-bond donor and acceptor is generally considered between 1.6 and 2.5 Å.[10] In protein-ligand complexes H-bonds were inferred when the distance was less than 3.5 Å.[11] Compared to van der Waals forces, salt bridges and H-bonds are strong non-covalent interactions[6] which could make important contributions to FXIII-A_2 dimer formation.

The dimer formation is crucial for the stability of FXIII-A_2 as two previous case reports on patients with congenital FXIII-A deficiency showed that impaired dimer formation makes FXIII unstable. In these case reports, the FXIII-A Arg260Cys and Tyr283Cys mutations were associated with impaired dimer assembly and decreased stability of mutant FXIII-A_2.[12,13] Our group has previously investigated the function of the activation peptide in stabilization of FXIII-$A_{2.}$ Our findings suggested that the amino acid sequence from Phe8 to Arg12 plays a crucial role in intersubunit interactions in the FXIII-A_2 dimer.[14] Those mentioned studies provided useful but limited information about the interactions that keep FXIII-A_2 stable. The FXIII 3D structure has been studied for many years, but the amino acid sequences or individual amino acid residues crucial for the integrity of the FXIII molecule, including FXIII-A_2 dimer formation, are still not well understood.

We have therefore undertaken a systematic approach that used *in silico* analysis of the FXIII-A_2 crystal structure to identify potentially crucial amino acid residues within the FXIII-A_2 dimer interface and to design their mutations, followed by single site-directed mutagenesis and cell expression studies to confirm which amino acid residues are vital for expression and stability of the FXIII-A protein.

Methods

Computer analysis methods and design of mutations

We used the FXIII-A_2 crystal structure 1F13.pdb[15] obtained from the Protein Data Bank in Europe (PDBe), and the PISA (Proteins, Interfaces, Structures and Assemblies) server (http://www.ebi.ac.uk/pdbe/pisa/) to identify the amino acid residues forming intersubunit salt bridges and H-bonds at the interface between two A-subunits. We further visualized the identified amino acid residues using Swiss-PdbViewer[16] (https://spdbv.vital-it.ch/) and evaluated salt bridges and H-bonds according to the distance and position between donor and acceptor atoms.

In order to investigate the effects of the identified amino acid residues on the stability of FXIII-A_2, we designed mutations using Swiss-PdbViewer. Principally, we changed the amino acid residues with charged side chains to amino acid residues without charge but of similar size, and this disrupted the electrostatic interactions without inducing significant steric changes so that the conformation should not significantly change after mutation.

For Lys257, we designed two mutations, Lys257Glu and Lys257Leu. The Lys257Glu mutation was identified in a FXIII deficient patient[17] but not further characterized *in vitro*, therefore we decided to express this mutation as well. Mutation of lysine, a polar amino acid with positive charge, into glutamic acid, a polar amino acid with negative charge, is expected to disrupt the interaction of Lys257-Glu401 by repelling electric forces, which could significantly influence the conformation of FXIII-A_2. Hence, we designed for our study another mutation, Lys257Leu. Leucine is a non-polar amino acid that would not support salt bridge or H-bond formation with the opposite A-subunit. Since its structure and molecular weight (131.17 g/mol) are similar to lysine (146.19 g/mol), we would not expect this mutation to significantly alter the conformation of FXIII-A.

For Arg100, we also designed two mutations, Arg100Gln and Arg100Leu. Arginine is a polar amino acid with positive charge. With the two mutations we aimed at testing its replacement by another polar (glutamine) and by a non-polar (leucine) amino acid.

We applied similar considerations in the design of the following mutations: Glu111Leu, Asn112Val, Lys113Leu, Asp343Val, Glu401Leu, and Asp404Val. According to visualization in PdbViewer, we expected all these mutations to disrupt the salt bridge and/or H-bond interactions between the two A-subunits, without major conformational changes within the FXIII-A monomers.

Wild-type and mutant F13A expression plasmids

We used the pcDNA5/FRT expression plasmid containing the human wild-type F13A cDNA sequence (pcDNA5/FRT-WT-F13A) that we had cloned previously.[14] We constructed the mutant F13A expression plasmids by site-directed mutagenesis of the wild-type expression plasmid with the GeneArt® Site-Directed Mutagenesis PLUS Kit (Invitrogen). We designed the mutagenic primers using the web-based GeneArt® Primer and Construct Design Tool (http://www.thermofisher.com/order/oligoDesigner). The primers are listed in Table 1.

We performed the mutagenesis reaction according to the manufacturer's instruction using One Shot® MAX Efficiency® DH5α™-T1R competent cells (Invitrogen). We purified the plasmids using the PureLink™ HiPure Plasmid Midiprep Kit (Invitrogen) and then used the sequencing service by Microsynth (Balgach, Switzerland) with standard CMV-forward and BGH-reverse primers. The plasmids with correct sequences were chosen for transfection.

Stable expression of wild-type and mutant FXIII-A proteins

We co-transfected Flp-In™ Chinese hamster ovary (CHO) cells (Invitrogen) with the pOG44 plasmid encoding a specific recombinase and one of our expression plasmids (wild-type FXIII-A or mutant FXIII-A plasmids) using Lipofectamine™ 3000 Transfection Reagent (Invitrogen) according the manufacturer's protocol. We cultured the transfected cells with 600 μg mL^{-1} hygromycin (Invitrogen) for selection. After culture for three weeks, stable cell lines for FXIII-A expression were obtained. Cell lysates were prepared as described below and stored frozen until further analysis.

Analysis of wild-type and mutant FXIII-A proteins

We employed ELISA, Western blotting, and a FXIII activity assay to analyze the cell lysates as described previously.[18] Cell lysates from untransfected cells were used as negative control.

For ELISA, we lysed the cells in a non-denaturing buffer (140 mM NaCl, 10 mM HEPES, pH 7.4, 1% Triton X-100, containing protease inhibitor cocktail without EDTA (Pierce, Thermo Fisher Scientific)). We diluted the cell lysates 1:50, 1:100 and 1:200 in Tris-buffered saline (TBS) (140 mM NaCl, 40 mM Tris, pH 7.4) containing 0.1%

bovine serum albumin (BSA). The assay was performed according to the protocol published previously.[19] The standard curve was obtained with serial dilutions of recombinant FXIII-A_2 (Zedira, Darmstadt, Germany). We measured the total protein concentration of the cell lysates using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's protocol, and calculated the FXIII-A expression per 100 µg of total protein.

For Western blotting, the cells were lysed directly in 1X SDS–Laemmli sample buffer (Bio-Rad, Hercules, USA) (about 200 µL/10^6 cells). Proteins were separated by electrophoresis on Bolt™ 8% Bis-Tris Plus Gels (Invitrogen) with 1X Bolt™ MES SDS Running Buffer (Invitrogen), and transferred onto Immun-Blot® PVDF membrane (Bio-Rad) with 1X Bolt™ Transfer Buffer (Invitrogen). We incubated the membrane for three hours at room temperature with a primary rabbit monoclonal anti-FXIII-A antibody (ab179444; Abcam, Cambridge, UK) diluted 1:2,000, followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (31460; Thermo Fisher Scientific) diluted 1:10,000, and development with the WesternBright Quantum HRP chemiluminescent substrate (Advansta, Menlo Park, USA). Finally, we visualized the membrane with a Fusion Solo S imaging system (Vilber, Marne-la-Vallée, France).

We measured FXIII transglutaminase activity of wild-type and FXIII-A mutants using a biotin incorporation assay as described previously.[18,20] Briefly, we loaded cell lysates containing 12 ng of wild-type or mutant FXIII-A, diluted in TBS (40 mM Tris, 140 mM NaCl, pH 7.4), onto the fibrinogen-coated plate and added the reaction mix containing thrombin, calcium, and the FXIII substrate biotinpentylamine. The reactions were stopped with EDTA at different time points (3, 10, 20, 30 and 40 min) and incorporated biotinpentylamine was detected via alkaline-phosphatase-labelled streptavidine. The assay was performed in duplicates in three separate experiments, using two different cell lysates from two separate expression experiments. We calculated the initial slopes of the activity over time curves for the time intervals of 3-10 min and 3-20 min as change of optical density per minute (ΔOD/min) and compared the FXIII-A variants with the wild-type using a t-test (IBM© SPSS© Statistics, version 26.0, IBM Corporation, Armonk, New York, USA).

Results

In silico analysis of the FXIII-A_2 crystal structure suggests ten amino acid residues that form five particularly strong interactions between two A-subunits

In a first step, our aim was to identify *in silico* the amino acid residues that may be crucial for FXIII-A_2 dimer formation. We identified 12 amino acid residues forming salt bridges and 21 amino acid residues forming H-bonds, among them ten are involved in both salt bridge and H-bond formation (Figure 1). Therefore, we supposed that these ten amino acid residues form the following five particularly strong interactions between the two A-subunits: Arg11-Asp343, Glu111-His450, Lys113-Asp367, Lys257-Glu401, Arg260-Asp404 (Figure 1). As an estimate of the strength[21] of these five interactions, we measured the distance between donor and acceptor atoms as shown in Table 2.

The ten amino acid residues are localized along the interface between the two A-subunits and can be divided into three groups according to the protein domains involved (Figure 2). The first group maintains interactions between β-sandwich domain and core domain and comprises Glu111 and Lys113. Together with Asn112 and Arg100, which are only involved in H-bond formation, these four amino acid residues of the β-sandwich domain of one monomer interact with Asp447, His450, Asp351 and Asp367 in the core domain of the opposite monomer. The second group of amino acid residues maintain interactions between the two core domains of opposite FXIII-A monomers. This group comprises Arg260 and Lys257 which interact with Asp404 and Glu401, respectively, in the core domain of the opposite A-subunit. The third group maintains interactions between core domain and activation peptide and comprises Asp343 of the core domain that interacts with Arg11 and Arg12 of the opposite activation peptide. From this *in silico* analysis we concluded, that these three groups of amino acid residues are crucial for maintaining the interactions between two A-subunits.

In a second step, we designed mutations of the amino acid residues that are putatively important for maintaining the FXIII-A_2 dimeric structure and hence stability. Our aim was to disrupt the interactions by introducing single mutations that do not support the formation of salt bridges and/or H-bonds. At the same time we took care to choose amino acids of similar size in order to prevent significant conformational changes due to steric hindrance alone. Of the five pairs of amino acid residues forming the five strong interactions described above, we mutated at least one amino acid residue of each pair.

Mutations of five amino acid residues abolished the expression of properly folded, soluble FXIII-A in CHO cells

In the next step, the FXIII-A mutants were stably expressed in CHO cells. As shown by the ELISA and Western blot results in Figure 3, we were unable to detect the expression of properly folded and soluble FXIII-A in case of mutations at Lys257 (Lys257Glu and Lys257Leu), Lys113Leu, Asp343Val, Glu401Leu, and Asp404Val. The mutants Glu111Leu (86 % ± 8 %), Asn112Val (118 % ± 3 %), and Arg100Leu (97 % ± 5 %) were expressed to a similar extent as the wild-type, whereas Arg100Gln strongly reduced the expression to 32 % ± 7 %.

FXIII activity was altered in some FXIII-A variants

Finally, we investigated the activity of the FXIII-A variants using an incorporation assay. We did not find any detectable FXIII-A activity in the mutations at Lys257, Lys113, Asp343, Glu401, and Asp404, which is consistent with our ELISA results. FXIII activity over time of the wild-type and variants that were expressed to a measurable extent, i.e. Arg100Gln, Arg100Leu, Glu111Leu, and Asn112Val, is shown in Figure 4A. The initial activity rates, calculated as slope of the curves during the first 10 or 20 min are shown in Table 3. When we compared the initial slopes of the wild-type and mutants (as illustrated in Figure 4B), Arg100Gln and Arg100Leu variants showed a significantly reduced activity in the first 20 min compared with the wild-type ($p<0.001$). The Glu111Leu variant on the other hand showed a trend towards increased initial activity in the first 10 min ($p=0.067$).

Discussion

It is still not well understood how the FXIII-A_2 dimer is held together. There is some anecdotal evidence from patients with congenital FXIII-A deficiency for mutations that disrupted FXIII-A_2 dimer formation. A Tyr283Cys mutation identified in a FXIII-deficient patient was expressed in a megakaryoblastic cell line and found solely in monomeric form.[13] An Arg260Cys mutation identified in a FXIII-deficient patient was proposed to preclude the ionic interaction (salt bridge) between Arg260 and Asp404 on the opposite A-subunit.[22] When this mutant was expressed in yeast, expression levels were significantly reduced and indeed no FXIII-A_2 dimer was detected, but part of the unstable protein was present in monomeric form.[12] An Asp404His mutation was detected in heterozygous form in a FXIII-deficient patient. The protein was not expressed but molecular modelling predicted disruption of the stabilising interaction with Arg260 of the opposite A-subunit.[23] While the mutations mentioned above are all located in the core domain of the FXIII-A molecule, our group has shown previously that an Arg11Gln mutation in the activation peptide also abolished FXIII-A expression, possibly by disabling intersubunit interactions.[14] We were therefore wondering whether there was a “hot-spot” region within the interface between the two A-subunits of the FXIII-A_2 dimer that is of particular importance for intersubunit interactions, or whether rather individual amino acid residues scattered along the entire interface may be crucial for intersubunit interactions leading to dimer formation and ensuring protein stability.

In our systematic approach reported here we identified potential candidates of important amino acid residues by analyzing data from the published crystal structure. For confirmation we expressed FXIII-A variants where we had mutated the putatively crucial amino acid residues. Our results suggest that four intersubunit interactions are crucial for expression and stability of FXIII-A: Arg11-Asp343, Lys113-Asp367, Lys257-Glu401 and Arg260-Asp404. These interactions involve the activation peptide (Arg11), the β-sandwich domain (Lys113) and the core domain (Lys257, Arg260, Asp343, Asp367, Glu401, Asp404). That means that there is no “hot-spot” region for intersubunit interactions, but amino acid residues that significantly stabilize FXIII-A_2 are distributed over the interface and several protein domains. The β-barrel domains have been attributed a role in the enzymatic activity of FXIII by protecting the active site cysteine,[24] but do not contribute significantly to FXIII-A_2 dimer formation.

In our study, single mutations of Lys113, Lys257, Asp343, Glu401, and Asp404 abolished the expression of FXIII-A. We cannot completely rule out that any misfolded, insoluble or inactive protein was still expressed. We suggest, however, that the combination of different detection methods, i.e. sandwich ELISA with two different polyclonal antibodies, SDS PAGE followed by Western blotting with a monoclonal antibody, and the functional incorporation assay, makes it less likely that we have missed any misfolded, insoluble or inactive protein. The lack of FXIII-A in patients with Lys257Glu[17] and Asp404His[23] mutations, as well as several mutations of Arg260,[1] supports our findings and attributes clinical relevance to the interactions Lys257-Glu401 and Arg260-Asp404 that occur in close proximity to each other in the core domain.

Asp343 forms a salt bridge with Arg11 in the opposite FXIII-A subunit and we have shown here and previously[14] that mutations of either of these amino acid residues abolish functional FXIII-A expression. *In silico* analysis of the crystal structure also showed the possibility that Asp343 forms an ionic interaction with Arg12, with a distance between donor and acceptor residues of 3.29Å. However, the position of these two amino acid residues does not support H-bonds which contribute to strong salt bridges. We therefore conclude that Arg12 and Asp343 do not form a strong salt bridge that stabilizes the FXIII-A_2 dimer, which is supported by our previous finding that an Arg12Gln mutation had no influence on FXIII-A expression.[14]

In our study, single mutations of Arg100, Glu111, and Asn112 had no deleterious effect on FXIII-A protein expression, only Arg100Gln (but not Arg100Leu) reduced the expression level. We therefore conclude that the intersubunit interactions these amino acids are involved in, Arg100-Asp447, Glu111-His450, and Asn112-Asp367, are not essential for the stability of FXIII-A_2. The result for Glu111 was unexpected since a strong interaction of both ionic interaction and H-bond was suggested by the structural analysis. However, the distance between Glu111 and His450 is the longest distance of the five interactions we identified as potentially particularly important, and this could mean that Glu111-His450 may be in fact the weakest interaction among those five interactions. Mutations of Arg100 and Asn112 were predicted to disrupt H-bond interactions with Asp447 and Asp367, respectively, on the opposite A-subunit. However, disruption of a single H-bond interaction may not be enough to prevent intersubunit interactions and dimer formation. Similarly, the loss of the H-bond by the Arg100Gln mutation was not assumed as the reason for the decreased FXIII-A expression we observed. Since the Arg100Leu variant was expressed normally, we suppose that the Arg100Gln mutation may have caused other subtle changes to the structure of FXIII-A. Interestingly, both Arg100 variants displayed reduced FXIII activity although no conformational changes caused by the mutations were predicted. Possible explanations include stronger subunit interactions or reduced accessibility for thrombin or FXIII substrates. In case of the Glu111Leu variant, we observed slightly increased FXIII activity compared with the wild-type in the early time interval (3-10mins). The difference almost reached statistical significance, suggesting that Glu111Leu may be activated faster than wild-type FXIII-A. We hypothesize that this mutation has a mild weakening effect on intersubunit interactions which may facilitate the dissociation during activation of FXIII-A. This may be in line with a recent study by Anokhin et al.[25] who showed new evidence that FXIII activation may involve dissociation of FXIII-A_2 into monomers. This would raise the question, how the postulated strong intersubunit interactions stabilizing the dimer would be broken during FXIII activation. In case of the Arg11-Asp343 interaction, proteolytic cleavage and subsequent release of the activation peptide[26] would obviously disrupt the Arg11-Asp343 interaction. Analytical ultracentrifugation studies showed that thrombin cleavage of a single activation peptide is sufficient to weaken intersubunit interactions and initiate the transition from dimer to monomer.[25] We can only speculate on the mechanisms of how the other three putatively strong and stabilizing interactions Lys113-Asp367, Lys257-Glu401 and Arg260-Asp404 would be broken during FXIII activation. In their article on structural and functional aspects of FXIII, Komaromi et al.[2] discuss the conformational changes that occur during the FXIII activation process. The stretch of amino acids Met350-Lys366 is part of the hinge region of the core domain that allows movement of the β-barrel domains, and Asp367 is located right next to that region. An increase in solvent accessible surface area upon activation has been described for the region spanning amino acids 248–264, indicating that this region also undergoes significant conformational changes during activation. These conformational changes could disrupt the interactions Lys113-Asp367, Lys257-Glu401, and Arg260-Asp404, possibly contributing to dimer dissociation and transition to the monomeric form.

In our study, we took a systematic approach to investigate amino acid residues that are involved in intersubunit interactions. However, we did not express variants of all the amino acids identified in our *in silico* analysis, but we focused on the putatively strongest combined ionic interaction and H-bond interactions and expressed at least one variant of every interaction pair. In addition, as proof-of-concept, we also expressed some variants of amino acids involved in H-bonds only. Hydrophobic interactions are also important interactions for maintaining protein structures, but these interactions are rarely single atom interactions so we did not take those into account.

A limitation of our study is that we cannot confirm that the undetectable FXIII-A variants would indeed exist as monomers only due to the disrupted intersubunit interactions, and hence we cannot confirm a causal link between the lack of expression of a mutant protein and the potential failure to form dimers. Failure to form a dimer has been reported for the Arg260Cys and Tyr283Cys mutations found in patients with congenital FXIII deficiency. Although small amounts sufficient to be analysed were expressed *in vitro*, those monomeric forms were unstable[11,12], which supports our hypothesis.

In summary, we have presented four FXIII-A intersubunit interactions, Arg11-Asp343, Lys113-Asp367, Lys257-Glu401 and Arg260-Asp404, that may be essential for the expression and stability of FXIII-A_2. Our findings are supported by reported mutations at Lys257, Arg260, and Asp404 found in patients with congenital FXIII-A deficiency. Our study contributes to a better understanding of the crucial elements that hold the FXIII molecule together.

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Authorship Contributions

Contribution: H.P.K. and V.S. designed the research; B.L. performed the experiments; B.L. and V.S. analyzed the results; B.L. and V.S. wrote the manuscript; H.P.K. reviewed the manuscript.

Disclosure of Conflict of Interest

The authors declare no conflicts of interest.

Data Sharing

For original data, please contact the corresponding author.

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Tables

Table 1. Primers for site-directed mutagenesis of the wild-type expression plasmid (pcDNA5/FRT-WT-F13A). The mutated codons are labeled with gray color and underlines.

Mutation	Sequence 5'→ 3'
Arg100Leu	Forward primer: 5'- AGAAGGGATCTCTTC<u>CTG</u>GTGGAATACGTCAT -3'
	Reverse primer: 5'- ATGACGTATTCCAC<u>CAG</u>GAAGAGATCCCTTCT -3'
Arg100Gln	Forward primer: 5'- AGAAGGGATCTCTTC<u>CAG</u>GTGGAATACGTCAT -3'
	Reverse primer: 5'- ATGACGTATTCCAC<u>CTG</u>GAAGAGATCCCTTCT -3'
Glu111Leu	Forward primer: 5'-GGTCGCTACCCACAG<u>CTG</u>AACAAGGGAACCTA-3'
	Reverse primer: 5'- TAGGTTCCCTTGTT<u>CAG</u>CTGTGGGTAGCGACC-3'
Asn112Val	Forward primer: 5'- CGCTACCCACAGGAG<u>GTC</u>AAGGGAACCTACAT -3'
	Reverse primer: 5'- ATGTAGGTTCCCTT<u>GAC</u>CTCCTGTGGGTAGCG -3'
Lys113Leu	Forward primer: 5'- TACCCACAGGAGAAC<u>CTG</u>GGAACCTACATCCC -3'
	Reverse primer: 5'- GGGATGTAGGTTCC<u>CAG</u>GTTCTCCTGTGGGTA -3'
Lys257Leu	Forward primer: 5'- AGAGGGAATCCCATC<u>CTA</u>GTCAGCCGTGTGGG -3'
	Reverse primer: 5'- CCCACACGGCTGAC<u>TAG</u>GATGGGATTCCCTCT -3'
Lys257Glu	Forward primer: 5'- AGAGGGAATCCCATC<u>GAA</u>GTCAGCCGTGTGG -3'
	Reverse primer: 5'- CCACACGGCTGAC<u>TTC</u>GATGGGATTCCCTCT -3'
Asp343Val	Forward primer: 5'- ATTTCTCTGCCCAT<u>GTT</u>AATGATGCCAATTT -3'
	Reverse primer: 5'- AAATTGGCATCATT<u>AAC</u>ATGGGCAGAGAAAT -3'
Glu401Leu	Forward primer: 5'- GACAGCACCCCCCAG<u>CTA</u>AATAGCGATGGCAT -3'
	Reverse primer: 5'- ATGCCATCGCTATT<u>TAG</u>CTGGGGGGTGCTGTC -3'
Asp404Val	Forward primer: 5'- CCCAGGAAAATAGC<u>GTT</u>GGCATGTATCGGTG -3'
	Reverse primer: 5'- CACCGATACATGCC<u>AAC</u>GCTATTTTCCTGGG -3'

Table 2. The distance between donor and acceptor atoms of the amino acid residues involved in five particularly strong interactions.

Amino acid interactions	The interacting residues and atoms	Distance (Å)
Lys257-Glu401	Lys257[NZ]-Glu401[OE1]	2.56
Arg11-Asp343	Arg11[NH1]-Asp343[OD2]	2.58
	Arg11[NH2]-Asp343[OD1]	2.98
Arg260-Asp404	Arg260[NE]-Asp404[OD1]	2.71
	Arg260[NH2]-Asp404[OD2]	3.24
Lys113-Asp367	Lys113[NZ]-Asp367 [OD1]	3.05
Glu111-His450	Glu111[OE1]-His450[NE2]	3.26

Table 3. Initial slopes of the activity-over-time curves.

FXIII-A variant	Slope 3-10 min, ΔOD/min	p-value	Slope 3-20 min, ΔOD/min	p-value
Wild-type	0.0403 (0.0116)	-	0.0443 (0.0051)	-
Arg100Gln	0.0291 (0.0239)	n.s.	0.0207 (0.0049)	< 0.001
Arg100Leu	0.0249 (0.0224)	n.s.	0.0213 (0.0034)	< 0.001
Glu111Leu	0.0585 (0.0124)	0.067	0.0457 (0.0073)	n.s.
Asn112Val	0.0343 (0.0418)	n.s.	0.0427 (0.0081)	n.s.

Data are shown as mean (SD). The p-value describes the comparison with the wild-type tested by t-test. n.s. = not significant

Figure Legends

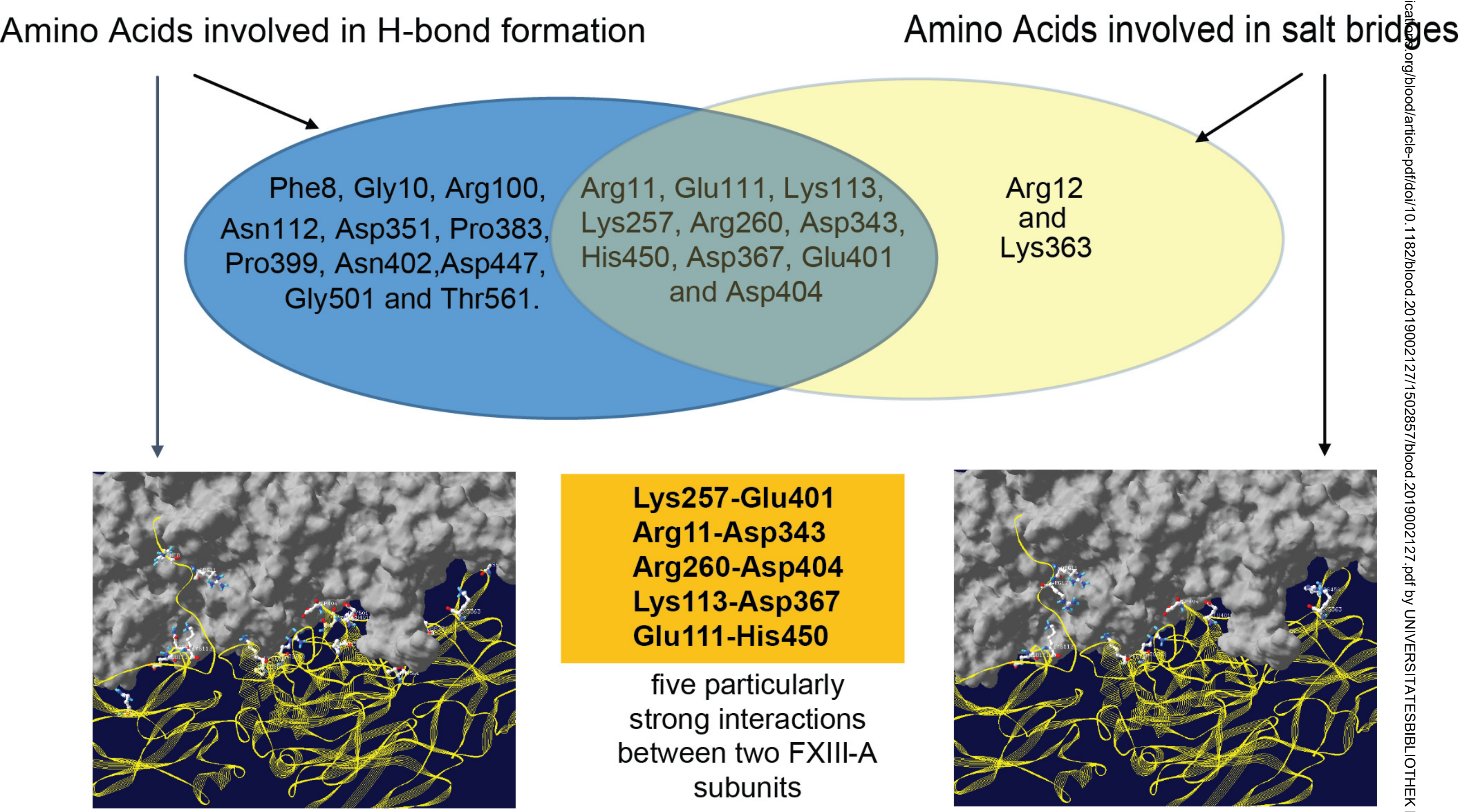
Figure 1. Amino acid residues of the FXIII-A_2 intersubunit interface involved in intersubunit interactions. Amino acid residues of the FXIII-A monomer shown as yellow ribbon are depicted against the grey space-filled model of the opposite FXIII-A monomer. Ten amino acid residues are involved in salt bridge and H-bond formation and suggested to form five particularly strong interactions (highlighted in the orange box) between the two A-subunits.

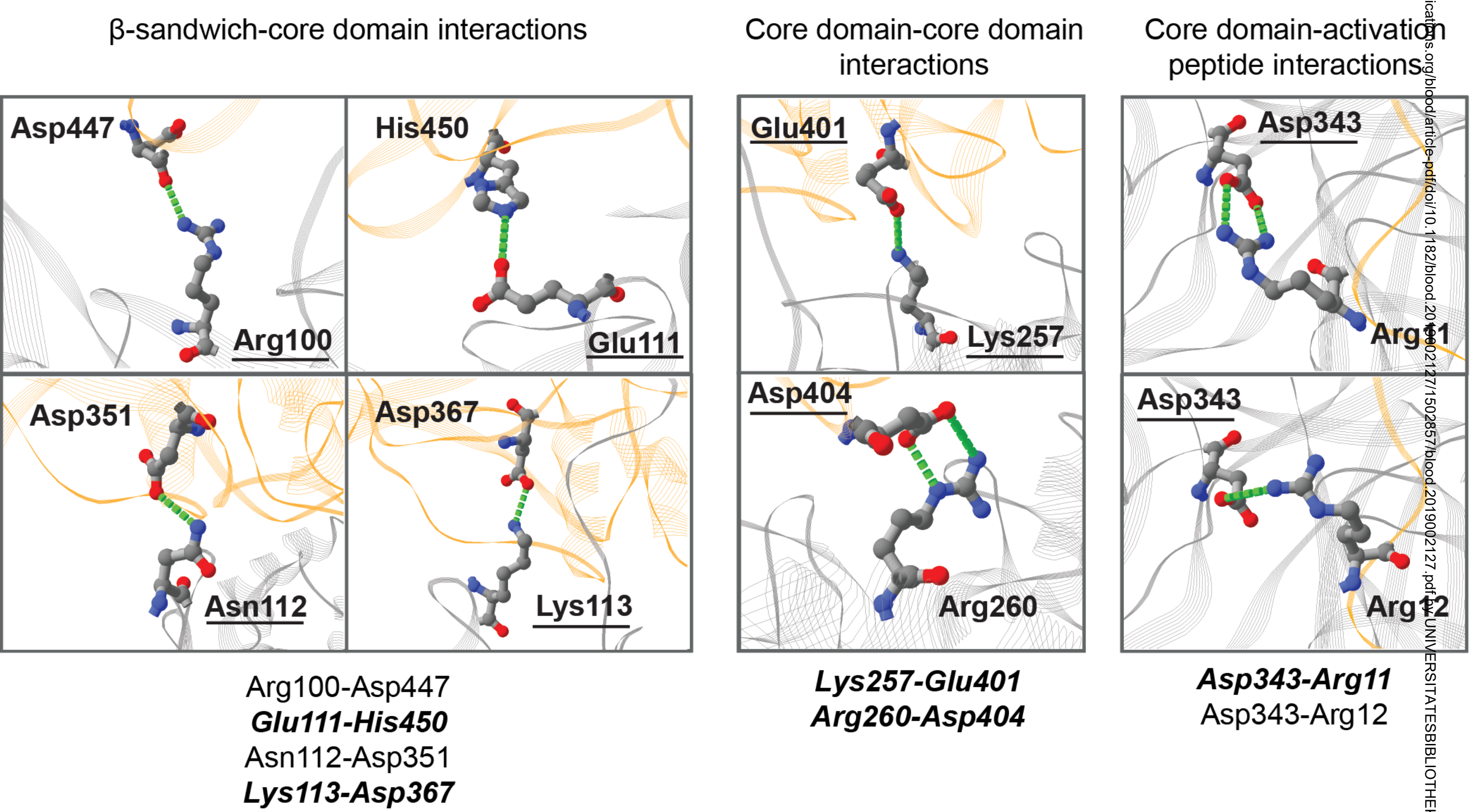
Figure 2. Detailed location of amino acid residues involved in intersubunit interactions. The amino acid residues can be grouped according to their protein domain and mediate interactions (indicated as dashed green lines) between the β-sandwich and core domain, between core domain and core domain, and between the activation peptide and core domain. The protein backbone of the first A-subunit is shown as grey ribbon, the backbone of the second A-subunit is shown as orange ribbon. The underlined amino acid residues were mutated to disrupt the interactions. The five amino acid pairs assumed to form particularly strong interactions are written in bold italic font.

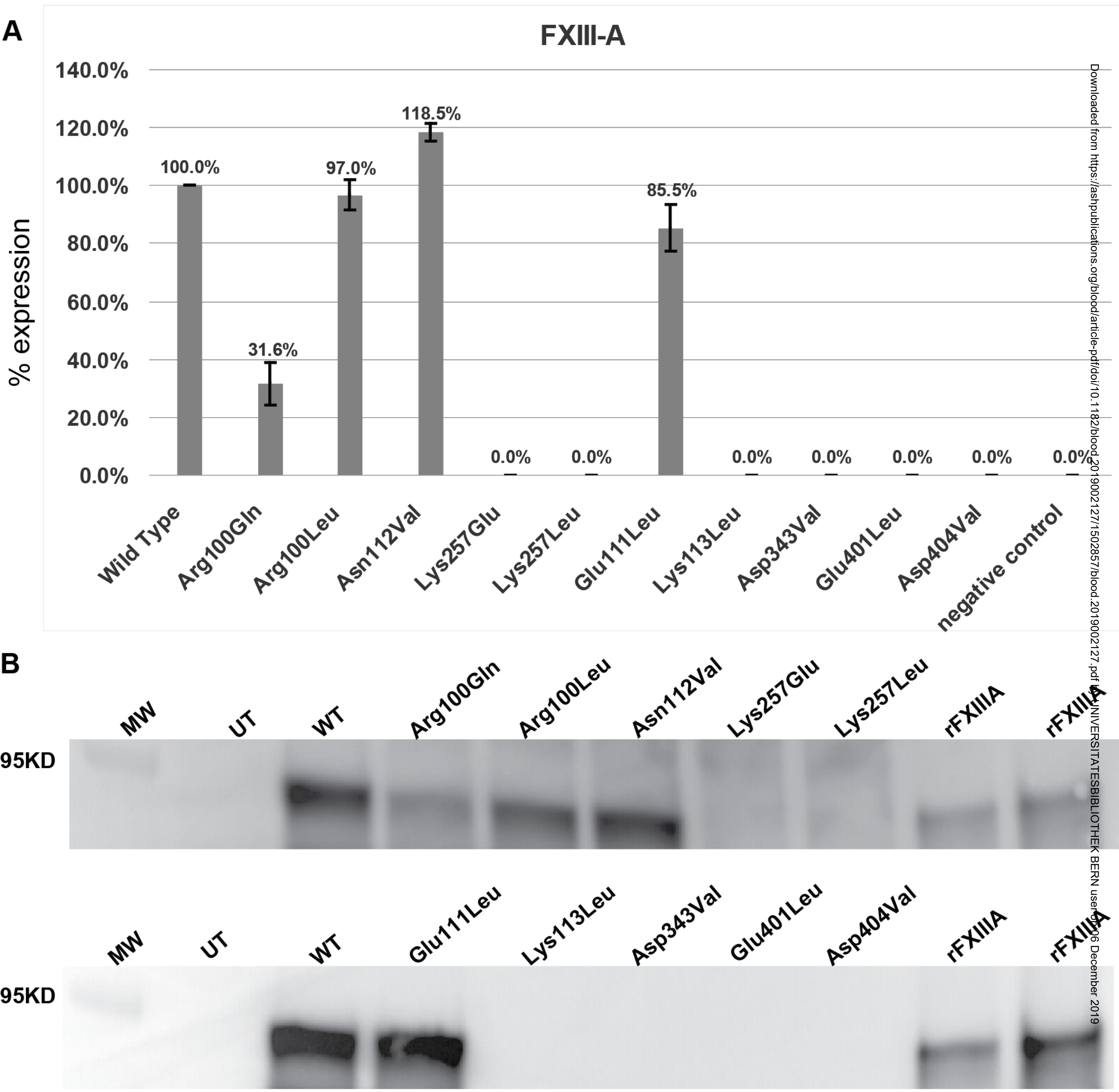
Figure 3. Protein expression of FXIII-A variants. (A) ELISA results showing the expression of the FXIII-A variants relative to the wild-type FXIII-A. Data are shown as mean from three experiments with error bars representing standard deviation. (B) Western blot showing the FXIII-A variants detected with a monoclonal anti-FXIII-A antibody. Controls include a molecular weight marker (MW), lysate from untransfected CHO cells (UT) as negative control, lysate from CHO cells transfected

with wild-type FXIII-A (WT), and commercially available rFXIII-A (Zedira) as positive control (20 ng and 50 ng loaded).

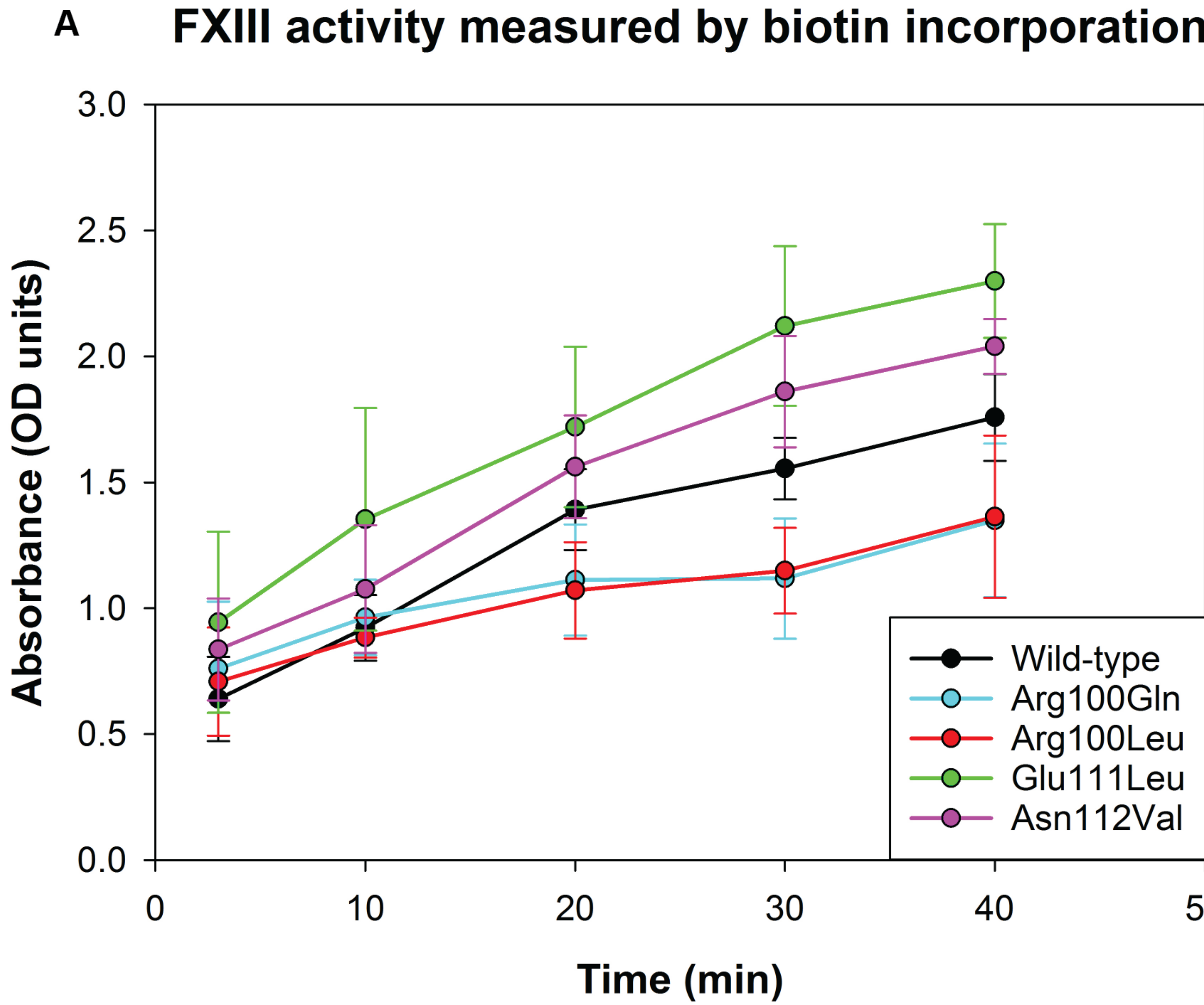
Figure 4. FXIII activity measured with an incorporation assay. Upon FXIII activation with thrombin and Ca^{2+}, transglutaminase activity was measured for up to 40 min. (A) FXIII activity of the wild-type and variants that were expressed to a measurable extent, i.e. Arg100Gln, Arg100Leu, Glu111Leu, and Asn112Val, is shown as absorbance over time (mean OD values ± standard deviation). The values are from three separate experiments. (B) The slopes of the activity curves during the first 10 min (dark grey bars) and 20 min (light grey bars) were calculated as ΔOD/min. Data are depicted as mean ± standard deviation. The asterix indicates statistical significance ($p<0.001$).


Amino Acids involved in H-bond formation
Amino Acids involved in salt bridges
Phe8, Gly10, Arg100, Asn112, Asp351, Pro383, Pro399, Asn402,Asp447, Gly501 and Thr561.
Arg11, Glu111, Lys113, Lys257, Arg260, Asp343, His450, Asp367, Glu401 and Asp404
Arg12 and Lys363
Lys257-Glu401
Arg11-Asp343
Arg260-Asp404
Lys113-Asp367
Glu111-His450
five particularly strong interactions between two FXIII-A subunits

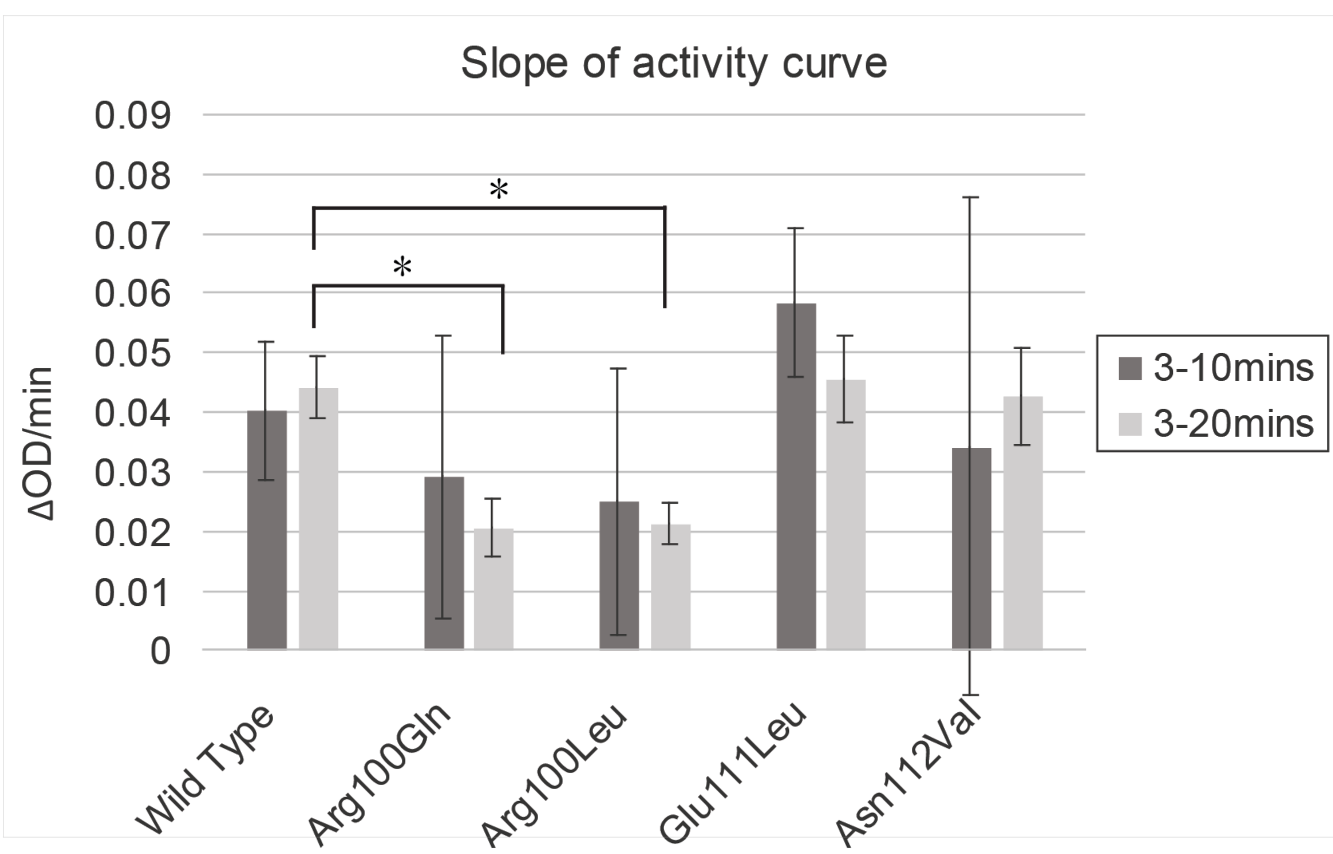

β-sandwich-core domain interactions
Core domain-core domain interactions
Core domain-activation peptide interactions
Asp447
Arg100
His450
Glu111
Asp351
Asn112
Asp367
Lys113
Glu401
Lys257
Asp404
Arg260
Asp343
Arg11
Asp343
Arg12
Arg100-Asp447
Glu111-His450
Asn112-Asp351
Lys113-Asp367
Lys257-Glu401
Arg260-Asp404
Asp343-Arg11
Asp343-Arg12


A
FXIII-A
% expression
140.0%
120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
100.0%
31.6%
97.0%
118.5%
0.0%
0.0%
85.5%
0.0%
0.0%
0.0%
0.0%
0.0%
Wild Type
Arg100Gln
Arg100Leu
Asn112Val
Lys257Glu
Lys257Leu
Glu111Leu
Lys113Leu
Asp343Val
Glu401Leu
Asp404Val
negative control
B
MW
UT
WT
Arg100Gln
Arg100Leu
Asn112Val
Lys257Glu
Lys257Leu
rFXIIIA
rFXIIIA
95KD
MW
UT
WT
Glu111Leu
Lys113Leu
Asp343Val
Glu401Leu
Asp404Val
rFXIIIA
rFXIIIA
95KD

A


FXIII activity measured by biotin incorporation
Absorbance (OD units)
Time (min)
Wild-type
Arg100Gln
Arg100Leu
Glu111Leu
Asn112Val
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
10
20
30
40
50

B


Slope of activity curve
ΔOD/min
3-10mins
3-20mins
*
*
Wild Type
Arg100Gln
Arg100Leu
Glu111Leu
Asn112Val
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09